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BILE ACID METABOLISM IN HUMANS: REGULATION OF SYNTHESIS AND CIRCADIAN RHYTHMS

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Bile acid metabolism in humans: regulation of synthesis and circadian rhythms

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**.....And mankind have not been given of knowledge
except a little**

The Holy Quran

To my family

ABSTRACT

Interfering with bile acids (BAs) signaling within the enterohepatic circulation (EHC) has recently emerged as an important way of controlling human metabolic homeostasis. This is highly relevant to a number of frequent disease entities such as dyslipidemia, fatty liver disease, insulin resistance, obesity, type 2 diabetes, gallstone disease and BA-induced diarrhea. The studies presented in this thesis focus on exploring how changes in the fluxes of BAs in the EHC may influence the synthesis and turnover of BAs and cholesterol, the metabolic signaling by endocrine fibroblast growth factors (FGFs) 19 and 21, and the modulation of lipid and carbohydrate metabolism.

Paper I presents a series of experiments characterizing the normal diurnal rhythms of BA EHC in 8 healthy males, and how they relate to BA and cholesterol synthesis. Responses to interruption of the EHC following treatment with cholestyramine, with and without adding statin, were examined in the same individuals. We identified an important difference between the diurnal rhythms of conjugated and unconjugated BAs, and could show that the transintestinal flux of conjugated BAs is responsible for changes in FGF19 secretion and regulation of BA production. We established that there is a previously unrecognized nocturnal influx of unconjugated BAs, which does not influence FGF19 or BA synthesis.

Paper II describes the relative contribution of hepatic vs intestinal FXR signaling in the regulation of BA synthesis in 57 healthy males, treated with 7 ascending doses of the potent non-steroidal FXR agonist Px-102 or placebo. Px-102 increased the level of circulating FGF19 in a dose dependent manner, while BA synthesis was almost completely suppressed already at the lowest dose, without increasing FGF19. Suppression of BA synthesis occurred before FGF19 levels were increased, indicating that activation of hepatic FXR is a major pathway for downregulation of BA synthesis in humans.

Paper III reports the effects of 55 hours of sleep deprivation or 66 hours of starvation on BA and lipid metabolism in 12 healthy volunteers. Deprivation of food, but not of sleep, markedly suppressed BA synthesis, despite lower/unchanged serum FGF19. Sleep deprivation progressively reduced FGF19, despite unchanged BA levels, indicating the presence of an independent (central) regulation of FGF19.

Paper IV explores how modulation of BA metabolism may influence circulating levels of the metabolic regulator FGF21, utilizing samples from Papers I and II. Data established the presence of a circadian rhythm with a nocturnal peak in the basal state. Both treatment with cholestyramine and Px-102 reduced serum FGF21 levels, indicating that the intracellular concentration of BAs may influence its secretion.

In conclusion, our experiments provide an ample characterization of the normal diurnal variation of the EHC of BAs, and of how its manipulation may influence human metabolism. This information is not only of significance for understanding normal physiology, but should also provide a foundation for later development of diagnostic and therapeutic principles to be applied on highly prevalent disease entities, including dyslipidemia, diabetes, fatty liver and cardiovascular disease.

LIST OF SCIENTIFIC PAPERS

- I. **Asynchronous rhythms of circulating conjugated and unconjugated bile acids in the modulation of human metabolism.**
Al-Khaifi A, Straniero S, Voronova V, Chernikova D, Sokolov V, Kumar C, Angelin B, and Rudling M.
J Intern Med (2018). doi: 10.1111/joim.12811 (2018).

- II. **An FXR agonist reduces bile acid synthesis independently of increases in FGF19 in healthy volunteers.**
Al-Khaifi A, Rudling M, and Angelin B.
Gastroenterology (2018). doi: 10.1053/j.gastro.2018.06.038 (2018).

- III. **Influence of food and sleep deprivation on bile acid metabolism in humans.**
Al-Khaifi A, Ståhle L, Angelin B, Rudling M, and Straniero S.
Manuscript (2018).

- IV. **Lowering of circulating FGF21 by modulation of bile acid metabolism in healthy males.**
Al-Khaifi A, Straniero S, Rudling M, and Angelin B.
Manuscript (2018).

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LIST OF ABBREVIATIONS

ASBT	Apical Sodium-Dependent Bile Acid Transporters
BAs	Bile Acids
BAAT	Bile Acid Coenzyme A:Amino Acid N-Acyltransferase
BSEP	Bile Salt Export Pump
BSH	Bile Salt Hydrolase
C4	7 α -Hydroxy-4-Cholestene-3-One
CA	Cholic Acid
CEM	Cholestyramine
CEM+STAT	Cholestyramine + Statin
CDCA	Chenodeoxycholic Acid
CYP27A1	Sterol 27-Hydroxylase
CYP7A1	Cholesterol 7 α -Hydroxylase
CYP8B1	Sterol 12 α -Hydroxylase
DCA	Deoxycholic Acid
EHC	Enterohepatic Circulation
FATP5	Fatty Acid Transport Protein 5
FFA	Free Fatty Acid
FGF19	Fibroblast Growth Factor 19
FGF21	Fibroblast Growth Factor 21
FGFR4	Fibroblast Growth Factor Receptor 4
FPLC	Fast Protein Liquid Chromatography
FXR	Farnesoid X Receptor
GC-MS	Gas Chromatography-Mass Spectrometry
GLP1	Glucagon-Like Peptide 1
HNF-4 α	Hepatic Nuclear Factor-4 α
HDL	High Density Lipoprotein
HMGC α AR	3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase
HPLC	High performance liquid chromatography
LCA	Lithocholic Acid
LC-MS-MS	Liquid Chromatography-Mass Spectrometry-Mass Spectrometry
LDL	Low Density Lipoprotein
LDLR	Low Density Lipoprotein Receptor
LRH-1	Liver Receptor Homolog-1
MCA	Muricholic Acids
NTCP	Sodium Bile Acid Co-Transporter
OATP	Organic Anion Transporting Polypeptide
OST α/β	Organic Solute Transporter A/B
PPAR α	Peroxisome Proliferator-Activated Receptor Alpha
SHP	Small Heterodimer Partner
SREBP2	Sterol Regulatory Element Binding Protein 2
TGs	Triglycerides
TGR5	Takeda G Protein-Coupled Receptor
UDCA	Ursodeoxycholic Acid
VLDL	Very Low Density Lipoprotein

1. INTRODUCTION

The importance of maintaining an efficient enterohepatic circulation (EHC) of bile acids (BAs) for securing normal bile secretion and intestinal fat absorption, as well as the critical role of BAs in the regulation of cholesterol balance, has been recognized for many years. More recently, it has been discovered that BAs also influence the homeostasis of lipid, glucose and energy metabolism, and interfering with BA signaling within the EHC has emerged as an important way of controlling human metabolic disease. This is highly relevant to a number of frequent disease entities such as dyslipidemia, fatty liver disease, insulin resistance, obesity, type 2 diabetes, gallstone disease, cholestasis, and BA-induced diarrhea. The studies presented in this thesis focus on exploring how changes in the fluxes of BAs in the EHC may influence the synthesis and turnover of BAs and cholesterol, the metabolic signaling by endocrine fibroblast growth factors (FGFs) 19 and 21, and the modulation of lipid and carbohydrate metabolism.

1.1 Cholesterol metabolism

Cholesterol is a steroid that constitutes an essential component of all cells in animals. Cholesterol also serves as precursor for a broad set of molecules including bile acids, steroid hormones and vitamins. De novo synthesis of cholesterol takes place in all body cells where the liver accounts for the highest production rate. This synthetic pathway involves a series of chemical reactions where the rate limiting enzyme is 3-hydroxy-3-methylglutaryl CoA reductase (HMGCoAR). Besides endogenous synthesis, body cells can obtain cholesterol from diet. Cholesterol transportation among various tissues is mediated through lipoprotein particles that transport hydrophobic lipids in the circulation. Five main classes of lipoprotein particles are present in the circulation; chylomicrons, very low density lipoproteins (VLDL), intermediate density lipoproteins (IDL), low density lipoproteins (LDL) and high density lipoproteins (HDL). Because it cannot be degraded, excess cholesterol in periphery is harmful for the cells and therefore it must be carried back to the liver where it can be converted into bile acids or excreted directly into the bile [1, 2].

Conversion of cholesterol into BAs in the liver provides a main excretory pathway for cholesterol, highlighting the importance of understanding the mechanisms of BA synthesis and excretion and the regulatory molecules involved in these pathways in humans.

1.2 Bile acids

Physiology

In contrast to the highly hydrophobic nature of cholesterol, BAs are characterized by their amphipathic structure, which enables them to function in the body aqueous environment. Within the body, BAs are predominantly localized within the

enterohepatic system where they serve different critical functions ranging from simple fat emulsifiers to potent modulators of gene expression. In the liver, BA secretion facilitates bile formation and drives the secretion of cholesterol and phospholipids into bile [3, 4]. BA production from cholesterol and their subsequent fecal excretion represents one of the major routes for cholesterol elimination from the body. Additionally, the bipolar nature of BAs enables them to form micelles which facilitate digestion and absorption of dietary lipids and fat soluble vitamins in the small intestine. Likewise, BA micelles in the gallbladder solubilize cholesterol in bile, preventing cholesterol precipitation and gallstone formation. Accordingly, disturbances in BA metabolism may lead to the development of different metabolic diseases, including cholestasis, gallstones, lipid malabsorption and atherosclerosis [5, 6].

Recent studies have found that BAs can act as signaling molecules capable of altering gene expression in fat, glucose and energy metabolism [5, 7, 8]. These responses are induced by modulating the activity of a number of receptors, including the nuclear farnesoid X receptor (FXR) [9-11] and the membrane Takeda G protein-coupled receptor (TGR5) [12]. An additional dimension of the influence of BAs is their ability to interact with gut microbiota which has been found to contribute to the maintenance of normal physiology [6, 13].

1.2.1 Synthesis and enterohepatic circulation of BAs

BAs in the liver

The biosynthesis of primary BAs from cholesterol is catalyzed by at least 17 enzymes located in different compartments within hepatocytes [14-16]. This occurs through two pathways, the classical and the alternative pathway (Figure 1), both of which modify the cholesterol steroid ring through several chemical reactions. The ultimate products of these reactions are the primary BAs, cholic acid (CA; 3 α ,7 α ,12 α -trihydroxy-5 β -cholanoic acid) and chenodeoxycholic acid (CDCA; 3 α ,7 α -dihydroxy-5 β -cholanoic acid). The major part of BA synthesis in humans liver occurs through the classical pathway, which starts with hydroxylating the 7 α -C of cholesterol by the rate limiting enzyme cytochrome P450 7A1, cholesterol 7 α -hydroxylase (CYP7A1). An intermediate of this reaction is 7 α -hydroxy-4-cholesten-3-one, known as C4, which is converted to CDCA unless it is further hydroxylated by sterol 12 α -hydroxylase (CYP8B1) producing CA. Thus, CYP8B1 activity determines the CA/CDCA ratio and hence the chemical and biological properties of the BA pool [7, 16]. The serum level of C4 has been found to synchronize with the activity of hepatic CYP7A1 and is considered to be a reliable biomarker for the rate of BA synthesis [17].

While only a small portion of BAs is produced by the alternative pathway in humans, both pathways contribute to BA production in mice. The alternative pathway is initiated by sterol 27-hydroxylase (CYP27A1) which in addition to hepatocytes is expressed in macrophages and other tissues [16]. Once synthesized, primary BAs are conjugated at

their side chain with either glycine or taurine. Conjugated BAs, referred to as bile salts (BSs), are then pumped into the bile canaliculi through the bile salt export pumps (BSEP) to be stored in the gallbladder. Following food intake, the gut hormone cholecystokinin stimulates gallbladder contraction causing the emptying of its bile into the duodenum and thereby initiating the postprandial cycles of enterohepatic circulation (EHC) of BAs [4, 16, 18].

BAs in the intestine

Within the intestinal lumen, BSs solubilize dietary lipids and fat-soluble vitamins facilitating their absorption by the intestinal epithelium. About 95-99% of BSs are actively and efficiently absorbed at the terminal ileum through the apical sodium-dependent bile acid transporter (ASBT) expressed in the apical membrane of ileal cells. Also, a portion of the BSs are passively absorbed in the upper small intestine [19]. Within the enterocytes, BSs are translocated to the basolateral side from where they are released into the portal blood through the heterodimeric organic solute transporter (Osta/Ost β) [20]. BSs that escape the reabsorption enter the colon where they are exposed to microbial modifications which further enrich the chemical diversity of BA pool by forming secondary BAs. The bacterial enzymes bile salt hydrolase (BSH) and 7 α -dehydroxylase deconjugate and dehydroxylate BAs, respectively. Successively, deoxycholic acid (DCA) is produced from CA while ursodeoxycholic acid (UDCA) and lithocholic acid (LCA) are formed from CDCA. Further reabsorption of BAs occurs passively in the colon [16] (Figure 1).

The absorbed primary and secondary BAs return to the liver through the portal vein and they are transported into the hepatocytes by sodium-dependent taurocholate cotransporting polypeptide (NTCP) and organic anion transporter (OATP). In the liver, the returned BAs are reconstituted, if required, and resecreted into the bile so completing one round of the EHC [4, 21]. Efficient uptake of BAs within the EHC results in the accumulation of BAs within the body forming the BA pool, which cycles between the intestine and liver 5-15 times/day. Despite of their efficient hepatic extraction, a portion of these BAs leaks into the circulation through liver sinusoids and can be seen as postprandial peaks in the peripheral circulation. In each round of BA EHC ~5% of the BA pool escapes reabsorption, and hence lost in feces. This fecal loss of BAs (~1 mmol/day) is compensated for by hepatic de novo synthesis from cholesterol, a process considered as one of the major routes for cholesterol excretion in humans and other mammals [4, 15, 22, 23].

The pivotal role of BAs to preserve normal homeostasis in humans is evident in conditions where BA synthetic enzymes or transporters are defective. Human patients with a homozygous deletion mutation in CYP7A1 are characterized by increased levels of circulating LDL cholesterol, hypertriglyceridemia, premature gallstone disease and premature coronary and peripheral vascular disease [24]. A mutation in the CYP27A1 gene causes a neurological disorder called cerebrotendinous xanthomatosis (CTX),

characterized by the accumulation of cholestanol in brain and tendon [25]. Mutations in hepatic BA transporters such as BSEP cause progressive familial intrahepatic cholestasis, a condition characterized by toxic levels of BAs in liver [26-28], and mutations in the ileal ASBT are associated with congenital diarrhea and steatorrhea [29].

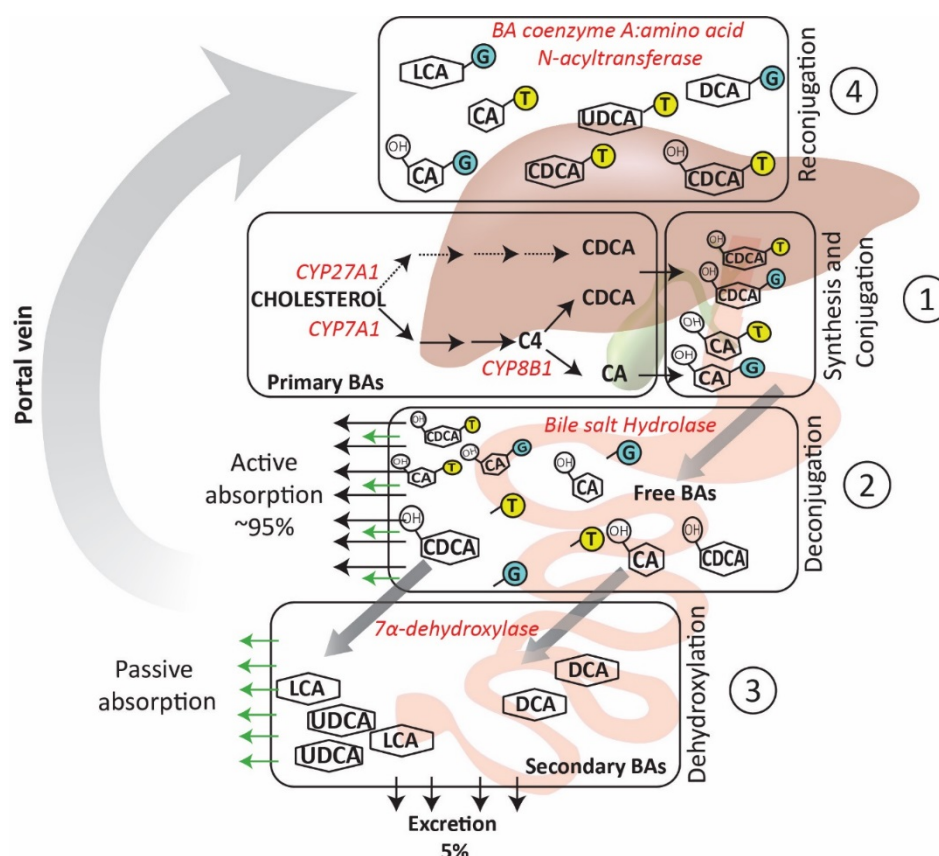


Figure 1. Synthesis and enterohepatic circulation of BAs. (1) In the liver, both the classic BA synthesis pathway, initiated by Cholesterol 7 α hydroxylase (CYP7A1) and the alternative pathway, initiated by CYP27A1, contribute to the synthesis of primary BAs; cholic acid (CA), which requires sterol 12 β -hydroxylase (CYP8B1), and chenodeoxycholic acid (CDCA). (2) When synthesized, BAs are conjugated to either taurine or glycine for secretion into bile. In the distal ileum, ~95% of BAs are actively absorbed and released into the portal blood. (3) Unabsorbed BAs are deconjugated by bacterial bile salt hydrolase (BSH) and are dehydroxylated by bacterial 7 α -dehydroxylase to form deoxycholic acid (DCA), lithocholic acid (LCA) and Ursodeoxycholic acid (UDCA). Approximately 95% bile acids are recycled and the 5% BAs that are fecally excreted are replenished by *de novo* BA synthesis. (4) BAs returned to the liver are reconjugated, by bile acid coenzyme A: amino acid N-acyltransferase (BAAT), before its secretion into the gallbladder.

1.2.2 Regulation of BA synthesis

Due to the cytotoxic effect of BAs, their levels within the body are tightly controlled through regulating both their synthesis and cellular transportation. For many decades BAs have been shown to regulate their own synthesis through feedback mechanisms [30], which are now evident to be mediated mainly through FXR. The latter is

predominantly expressed in liver and ileum and found to play central role in maintaining BA homeostasis [9, 10]. During their EHC, BAs activate both intestinal and hepatic FXRs resulting in feedback signaling that suppresses CYP7A1 gene expression [31]. The interaction of both intestinal and hepatic signaling in regulating BA synthesis in vivo complicates the exploration of the regulation of BA synthesis in humans, and much of our knowledge has had to rely on experiments in animal models.

Hepatic FXR pathway

Independently of FGF19, BAs inhibit their own synthesis by activating hepatic FXR which then induces the expression of an atypical nuclear receptor known as small heterodimer partner (SHP). SHP in turn negatively regulates CYP7A1 transcription by inactivating the heterodimerization of LRH-1 (Liver receptor homologue-1) and HNF-4 α (hepatic nuclear factor-4 α), a transcription factor complex required for the induction of CYP7A1 gene expression [32, 33]. Mice lacking SHP activity exhibit an increased basal expression of CYP7A1, nevertheless this animal model maintains the ability to suppress CYP7A1, which was suggested to be due to FGF15 activity [34]. This was supported by the upregulated CYP7A1 gene expression in intestine-specific FXR KO mice, an effect that was not observed upon the loss of hepatic FXR even when treated with an FXR agonist [31]. Animal experiments conducted so far indicated that inhibitory signaling from the intestine is favored over that in the liver in the context of CYP7A1 downregulation. On the other hand, the recent identification of a posttranscriptional mechanism on CYP7A1 mRNA, mediated by hepatic FXR, indicates that a hepatic mechanism may be elicited rapidly (within 30 minutes), and that the inhibitory role of FGF15 appears as later response [35].

Intestinal FXR pathway

This pathway is initiated following BA flux into the ileal cells where they bind and activate the intestinal FXR. Subsequently, the fibroblast growth factor 19 gene (FGF19: the human ortholog of FGF15 in mice) is expressed [36], resulting in the secretion of this protein into the portal vein to the liver. There it activates the membrane FGF receptor-4 (FGFR4) in complex with the co-receptor, β -klotho [36, 37]. This stimulates an intracellular signaling pathway which eventually suppresses BA synthesis [36, 38]. Although human FGF19 and mouse FGF15 are only ~50% homologous, their inhibitory effect on BA synthesis seems to be conserved [36, 38].

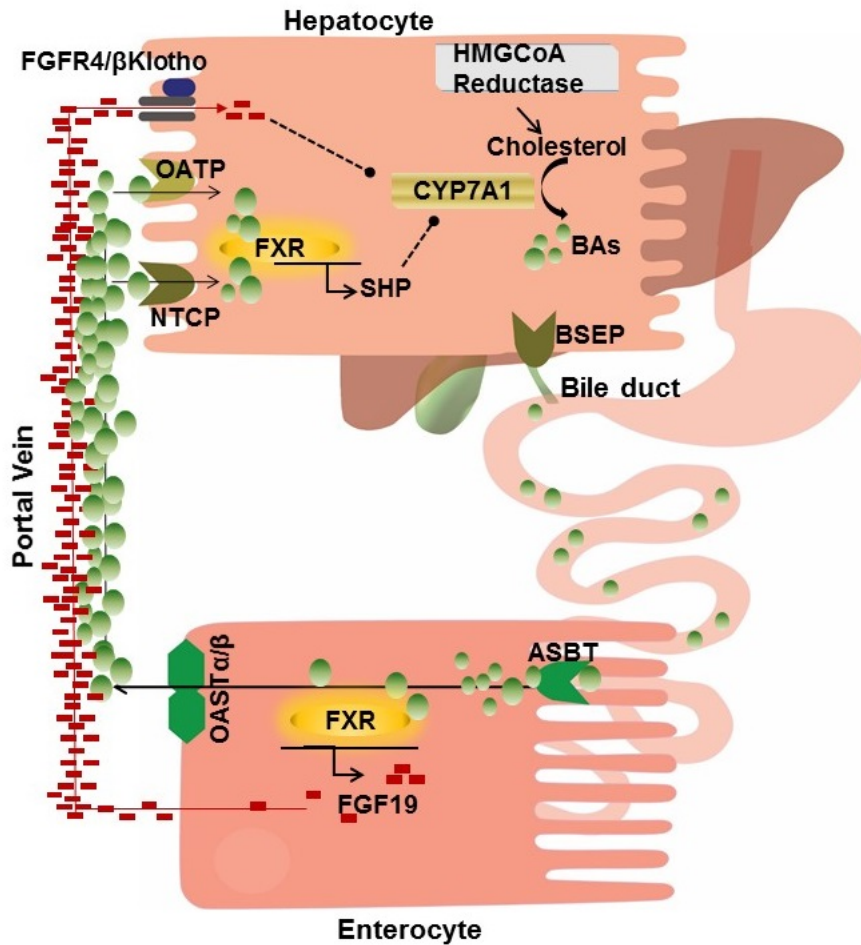


Figure 2. Regulation of BA synthesis. Regulation of BA synthesis through hepatic and intestinal pathways. BAs are synthesized in liver, stored in gallbladder and released into the gut following meal intake. In the ileum, BAs are reabsorbed through Apical sodium dependent BA transporter (ASBT) and activate FXR to induce secretion of FGF19 from enterocytes to reach the liver. FGF19 suppresses CYP7A1 through its receptor complex FGFR4/βKlotho. BAs enter the hepatocytes through their transporters Sodium-taurocholate cotransporting polypeptide(NTCP) and organic anion transport protein (OATP) to activate FXR. Hepatic FXR activation induces Small Heterodimer partner (SHP) transcription which in turn prevents the transcription factor HNF4α from binding and activating the CYP7A1 promoter, thereby inhibiting CYP7A1 gene expression.

FGF15/19 belongs to the large gene family of FGFs that play a fundamental role in cell growth, differentiation and embryonic development. The FGFs generally contain a heparin-binding domain that retains them attached to extracellular matrix components, facilitating their autocrine/paracrine signaling through their FGFRs. Together with FGF19, FGF21 and FGF23 form a separate subfamily of FGFs that lack this domain, which explains why they are found circulating in plasma and may act in a “hormone-like” fashion. FGF21 and FGF23 contribute to the control of glucose/lipid and phosphate/vitamin D metabolism, respectively. Another common feature of this subclass of FGFs is their requirement for a transmembrane protein linked to their receptor called β- or α-Klotho. This co-receptor stabilizes the FGF-FGFR binding in the absence of heparin-binding domain [39, 40].

Studies in transgenic and knockout mice of the FXR-FGF15- β -Klotho/FGFR4 pathway provide most of the current evidence for the vital role of FGF15 in mediating BA feedback regulation in mice liver. Knockouts of the aforementioned genes failed to suppress CYP7A1 expression and showed elevated BA synthesis [31, 36, 37, 41]. Similar disturbances were reported in mice following the ablation of ASBT, the intestinal bile acid transporter [42]. In contrast, repressed CYP7A1 gene expression and BA pool were observed in mouse models overexpressing hepatic FGFR4 or following administration of recombinant mouse FGF15 [43]. Infusing FGF19 in humans was constrained due to the carcinogenic effect observed in animals treated with this protein [44, 45]. Although a recently developed non-tumorigenic variant of FGF19 effectively inhibited BA synthesis, the doses used to induce such an effect were highly supra-physiological [46], in congruence with what has been observed in mice [47].

Additional roles of FXR in maintaining BA homeostasis

In addition to regulating CYP7A1 expression, FXR coordinates BA homeostasis by regulating the transcription of different BA transporter proteins within the EHC compartments. Both in liver and intestine, FXR stimulates BAs efflux by activating BSEP and Ost α/β and reduces BAs cellular uptake by suppressing NTCP and ASBT [21, 32, 33, 48]. The key role of FXR in maintaining BA physiology was highly supported by studies on FXR-knockout animals which displayed an increased BA synthesis and pool size [31]. Also, treating wild type mice with FXR agonists suppressed several genes of both classical and alternative synthetic pathways [49]. In humans, the physiological significance of FXR has been evaluated using FXR agonists, including natural BA ligands, e.g. CDCA and CA, and synthetic FXR agonists, e.g. GW4064, obeticholic acid, fexaramine and Px-102. BA binding resins, e.g. cholestyramine and colesvalam, have also been used as a tool to study BA-FXR signaling. These compounds are non-absorbable and act by binding to BAs in the intestinal lumen, blocking their intestinal uptake and leading to their excretion which eventually stimulates BA synthesis from cholesterol [50].

1.2.3 Properties of individual BAs

Different BAs have been shown to elicit diverse physiological responses and different receptor binding affinity. Thus, BAs cannot be considered as one entity, and accurate knowledge of BA composition, in addition to BA pool size, is required for a better understanding of human physiology and pathophysiology. The properties of BA chemical structure, including number of hydroxyl groups as well as their conjugation status, thus determine their biological activities [7]. Animal and in vitro experiments have shown that hydrophobic BAs such as CDCA and DCA are the most potent activators of FXR followed by the trihydroxylated CA [9-11]. In contrast, UDCA [51] and the rodent major

BAs, muricholic acids (MCAs), seem to antagonize FXR activity which highlights a key interspecies variation [52, 53]. Also, BA binding affinity to receptors such as TGR5 depends on their chemical structure [54]. Moreover, the conjugation status of BAs affects their binding affinity to their receptors [54] and influences their cellular uptake through different transporters. While the majority of conjugated BAs are efficiently transported by NTCP in humans, unconjugated BAs are actively reabsorbed by the organic anion transporting polypeptide (OATP) [55-57].

Furthermore, based on their hydrophobicity, BAs vary in their ability to form micelles. Among all BAs, CA - due to its strong hydrophilic characteristics - is the most potent in facilitating fat emulsification and absorption. For that reason, hypercholesterolemia is accelerated in mice when fed a high cholesterol diet together with CA, an effect that was not observed in CYP8B1 knock out animals [58].

1.2.4 Interspecies variation in BA metabolism

Most of our current understanding about the core pathways in BA metabolism is based on experiments in mice or rats. However, the existence of several marked interspecies variations should be considered when translating data from experiments in rodents into human situations [14]. Such species differences include; BA composition, synthesis and signaling pathways, gallbladder presence, and intestinal microflora; dissimilarities also exist regarding diurnal rhythms of BA and cholesterol metabolism [47].

The nature and composition of the BA pool is highly variable among humans and rodents. In humans it is known that the BA pool tends to be more hydrophobic, with CDCA and CA constituting a major and equal part of the pool (~40% each), followed by DCA (~20%); the majority of human BAs are glycine conjugates. On the other hand, the BA pool in mice is mostly composed of CA (~60%) and the unique mouse BAs, α - and β -muricholic acids (α -MCA and β -MCA) that largely exist in taurine conjugated form. The latter mouse BAs make up a highly hydrophilic BA pool characterized by poor detergent properties and FXR antagonizing activity [7, 16, 52, 53]. Such variations may increase the prevalence of BA-related diseases in humans, such as atherosclerosis and cholesterol gallstones, compared to rodents. Another important interspecies variation is the established variation in the circadian rhythms of cholesterol and BA syntheses. While the synthesis of both cholesterol and BAs occurs nocturnally in mice, they are asynchronous in humans, with cholesterol production peaking at night and BA synthesis around noon and late evening [59].

These differences between humans and rodents may partly be related to the different BA pool composition, with different capacities to influence FXR activity and hence CYP7A1 expression. Although the inhibitory signaling of FGF19 and FGF15 may be conserved [38], the proliferative effect of the former but not the latter makes such interspecies difference to be of great concern [45]. Accordingly, it is challenging to translate

knowledge on BA and cholesterol metabolism in animals to humans. Therefore, there is an important need for well-performed and highly standardized human experiments.

1.2.5 Role of BAs in regulating lipid and glucose metabolism

The role of BAs as metabolic regulators was established when favorable metabolic effects were unexpectedly observed following treatment with BA sequestrants. Disruption of the EHC by treatment with BA binding resins, such as cholestyramine, colestipol and colesevelam, has been effectively used for the treatment and prevention of coronary heart disease by lowering plasma cholesterol levels. However, a drawback of such treatment is the associated increase in plasma levels of triglycerides (TG). The promising effects of BAs sequestrants was also reported in type 2 diabetic patients that showed an improved glycemic control when treated with colesevelam [7, 8, 13, 60].

Another commonly used strategy to manipulate BAs EHC is the use of natural and synthetic FXR agonists which through modulating the activity of CYP7A1 lower TG plasma levels [61-63]. Moreover, this approach has been used as a potential therapy for the treatment of T2DM and nonalcoholic steatohepatitis [13]. FXR activation in rodent models was shown to reduce hepatic and plasma TG levels, and to improve glycemic control and insulin sensitivity [64, 65]. In contrary, FXR-deficient mice had elevated serum cholesterol, TG, LDL and VLDL in spite of the upregulated BA synthesis that should have facilitated higher elimination of cholesterol from the body [66].

Part of the metabolic effects of BAs may be mediated through TGR5, which is highly expressed in intestinal L cells and brown adipose tissue [12]. Activation of TGR5 by BAs induces the expression of the intestinal incretin, glucagon-like peptide 1 (GLP-1) which acts on pancreatic β cells to stimulate insulin release following a meal and hence regulates glucose homeostasis [67]. The promising effect of GLP-1 in glycemic control led to the development of GLP-1 analogues and TGR5 agonists for the treatment of diabetes [68]. TGR5 signaling may be a likely mechanism by which BAs exert their influence on energy homeostasis, as indicated by the stimulation of fatty acid oxidation and uncoupling proteins upon binding of BA to TGR5 expressed in the brown adipocytes [69].

1.3 FGF21

As already mentioned, FGF21 is another member of the FGF subfamily that exhibits “hormone-like” functional properties. It is highly expressed in the liver, and induced by the nuclear receptor peroxisome proliferator-activated receptor alpha (PPAR α), which is activated by FFAs in conditions of metabolic energy-deprivation [70-72]. FGF21 was found to signal to the adipose tissue and the brain through which it induces most of its positive metabolic effects. For its signal transduction, like FGF19, FGF21 requires the FGFR1c/ β -klotho complex [47, 73, 74].

About a decade ago, FGF21 was reported to improve glucose, insulin, and TG levels, and to reduce body weight in rodents and non-human primates [47, 75]. Unlike FGF19, no proliferative effect was observed when pharmacological doses of FGF21 were administered which accelerated the translation of such studies into humans [76, 77]. Clinical trials with FGF21 analogues demonstrated reduced body weight and plasma LDL cholesterol, TG and insulin, but with very minor reductions in glucose levels [78].

Interesting data from recent animal experiments have also demonstrated that FGF21 may also influence BA metabolism by directly controlling CYP7A1 gene expression [79-81]. Whether such associations exist in humans has not been reported yet.

2. AIMS

The main goal of this thesis is to understand the regulation of bile acid synthesis in humans and the physiological significance of FGF19 in this regulation. We therefore aimed to answer the following questions:

1. How do different BA species (conjugated/unconjugated) in humans relate to the activity of CYP7A1 and FGF19? Do they exhibit a similar diurnal variation?

Paper I

2. Is hepatic FXR activation the driving signal for the suppression of BA synthesis? To which extent does the intestinal FXR pathway contribute to this inhibitory signaling?

Paper II

3. What effects do sleep deprivation and prolonged fasting have on BA metabolism?

Paper III

4. Does modulation of BA synthesis change the circulating levels of FGF21?

Paper IV

catheter. Standardized meals (breakfast, lunch, dinner, supper) were provided at the indicated times (Figure 3), and they slept in darkness from 22:00 to 07:30. CME (4g; Questran, Bristol-Myers Squibb, Princeton, NJ) was given as an oral drink before each meal day 1. In the CME+STAT experiment, they were also given four daily doses of 40mg atorvastatin (Lipitor, Pfizer). The first and second doses were taken in the morning 2 days before CME administration, and the third and fourth in the morning days 1 and 2.

Paper II: Double-blind, randomized and placebo-controlled study. It included 54 healthy males randomized into 8 groups; placebo group (n = 14) and 7 treatment groups (n = 4 or n = 6). Drinking solutions of either placebo or one of 7 single ascending doses of Px-102 was given to the subjects. Px-102 doses were; (0.15, 0.3, 0.6, 1.12, 2.25, 3.38, or 4.5) mg/kg. Blood samples were collected over 24 hours at the following times; pre-dose (0 hours), 15 min, 30 min, 1, 1.5, 2, 4, 6, 8, 12, 24 hours after drug intake (Figure 3).

Paper III: The study included 12 healthy subjects (5 women and 7 men) who were randomly allocated into 2 groups and followed for 8 days. Based on a crossover design, each group was scheduled for 55 hours of sleep deprivation with unrestricted caloric intake and 66 hours of fasting followed by 24 hours of restricted caloric intake. The first group 'FS group' (3 women and 3 men) started with the food deprivation and the second group 'SF group' (2 women and 4 men) started with the sleep deprivation experiment. Blood samples were collected daily at 10 am with days 1 and 8 as baseline time points (Figure 3).

Paper IV: This study investigated the changes in serum FGF21 levels in response to perturbed BA synthesis experiments included in paper I and II.

3.2 Serum analyses

3.2.1 Cholesterol and triglycerides

Serum levels of total cholesterol and TG were determined using colorimetric reagents (Roche/Hitachi, Mannheim, Germany; TG 12016648, Chol 12016630). Samples of 5 µl were run in duplicate.

Cholesterol and TG within VLDL, LDL, and HDL particles as well as glycerol levels were measured by fast protein liquid chromatography (FPLC). Lipoproteins were separated by size-exclusion chromatography. Either cholesterol or TG reagent (cholesterol and TGs: Roche Diagnostics, GmbH, Mannheim, Germany) was continuously delivered into the eluate (the separated lipoproteins) followed by measurements of absorbance at 500nm. The lipid concentrations in the lipoprotein fractions were calculated as the product of the area percent of the individual chromatogram and the lipid content determined from the injected sample.

3.2.2 Assay of lathosterol (cholesterol synthesis)

Lathosterol was extracted from serum samples and analyzed with gas chromatography-mass spectrometry (GC-MS). Serum sample (25 μ l) was mixed with equal volume of internal standard, D₄-Lathosterol, and 500 μ l of isotonic NaCl and 3 mL Folch (CHCl₃:MeOH 2:1) and incubated overnight. The CHCl₃ phase was separated and evaporated at 60°C with nitrogen gas. The samples were then dissolved in 0.5 mL MeOH/H₂O 4:1 and isolated by Isolute MF-C18 0.1 g column. The samples were derivatized by silylation with (pyridine:hexametyldisilan:trimethylchlorosilane 3:2:1), trimethylsilane reagent, evaporated at 60°C under nitrogen and dissolved in hexan prior to GC-MS analysis. Levels of lathosterol were normalized for plasma cholesterol levels [82].

3.2.3 Assay of 7 α -hydroxy-4-cholestene-3-one (C4) (Bile acid synthesis)

Paper I: High performance liquid chromatography (HPLC) was used to measure C4 in serum samples, as described elsewhere [17]. Serum samples (0.5 mL) was diluted with saline, and 10 μ L 7 β -4-cholestene-3-one was added as internal standard. The samples were applied on C8 Isolute SPE columns (500 mg, 3 ml, International Sorbent Technology Ltd, Hengoed, UK), washed with 65% methanol and eluted with hexane:chloroform (95:5, v/v). The eluted product was dried by exposing to nitrogen at 60 °C and dissolved in 100 μ L of acetonitrile. Fifty μ L of acetonitrile were injected into the HPLC system at 20°C (mobile phase: acetonitrile/water 95:5; 1 mL/min) by using a Nova-Pak C18 steel column, 3.9 X 300-mm ID, 4 mm particle size (Waters Corp, Milford, MA). The wavelength was 241 nm. C4 values were corrected for total serum cholesterol [59].

Paper II & III: C4 was quantified in combination with serum BAs by liquid chromatography-tandem mass spectrometry (LC-MS/MS). All chemicals and solvents were of the highest purity available. Methanol, acetonitrile and formic acid were obtained from VWR (Radnor, Pennsylvania). Deuterium-labeled standards of C4 and unconjugated, glycine- and taurine-conjugated BAs were from Steraloids, Inc. (Newport, RI, USA). Serum sample (100 μ L) was mixed with 400 μ L of acetonitrile and 10 μ L of each of the deuterium-labelled C4 and BA internal standards (10 ng d₇-C4 and 25 ng d₄-BA) and vortexed for 5 sec. After 15 min centrifugation at 14,000 g, supernatants were transferred to glass tubes and dried under a stream of nitrogen at 40°C. The residue was dissolved in 200 μ L of MeOH-H₂O (1:1, v/v), and 5 μ L of this solution was injected.

C4 and individual BAs were analyzed by LC-MS/MS using a Xevo TQ-XS Triple Quadrupole Mass Spectrometer (Waters Corp., Mass., USA) operated in the ion evaporation mode with an ion spray ionization probe. Data were acquired and processed using MassLynx MS Software. Chromatography was achieved using a reverse-phase C18

column, 1.7 μ m, 2.1 \times 50 mm internal diameter (Waters Corporation, Mass, USA). The column flow was 0.5 mL/min. C4 levels were normalized for total cholesterol.

3.2.4 Bile acids

The 15 major human BAs (free, glycine-, and taurine-conjugated CA, CDCA, DCA, LCA, UDCA) were quantified in combination with C4 by LC-MS/MS as described in section 3.2.3 ‘‘Assay of C4’’

3.2.5 FGF19 and FGF21

A sandwich enzyme-linked immune sorbent assay (ELISA) was used for colorimetric detection of FGF19 and FGF21 in serum following the manufacturer’s instructions (FGF19 Quantikine ELISA kit, Cat. No. DF1900; R&D Systems, Minneapolis, MN, USA) and (FGF21 Quantikine ELISA kit Cat. No. DF1900; R&D Systems Minneapolis, MN, USA). All samples were assayed in duplicate; the intra- and inter-assay coefficients of variation were <10%.

3.2.6 Glucose and insulin analysis

Serum glucose was determined using an enzymatic colorimetric assay with reagents from Roche Diagnostics, Mannheim, Germany. Plasma insulin levels were analyzed using Mercodia ultrasensitive insulin ELISA assay for humans (Cat. No. 10-1132-01, Mercodia Developing diagnostics, Uppsala, Sweden)

3.3 Statistics

All statistical analyses are described in the corresponding papers I-IV. Graph Pad Prism software (GraphPad Software Inc., La Jolla, CA) or R commander were used for statistical analyses. In general, the designs of all experiments are based on examining 2 or more groups of participants ($n \leq 14$) who received different treatments and monitored over a period of time. Thus, significance of differences between control and treated groups or within a single group were analyzed using the equivalent nonparametric tests of either 2-way ANOVA, one-way ANOVA or paired t-test.

4. RESULTS

4.1 Paper I

Asynchronous rhythms of circulating conjugated and unconjugated bile acids in the modulation of human metabolism

Aims

This paper aimed to explore the normal diurnal rhythms of the EHC of BAs in healthy humans and how they relate to BA and cholesterol synthesis. In the same individuals, we characterized the effects of an interruption of the bile acid EHC using cholestyramine treatment (CME), and also to which extent these responses may be modified by simultaneously blocking hepatic cholesterol synthesis by adding statin (CME+STAT).

Findings

Conjugated and unconjugated BAs have asynchronous diurnal rhythms.

Analysis of serum BA conjugation in control subjects indicated that conjugated BAs exhibit a markedly different profile compared to unconjugated BAs. Conjugated BAs increased after meal intake and accounted for the observed postprandial peaks of total BAs. On the other hand, the levels of unconjugated BAs were not influenced by food intake, instead they displayed a robust increase at the end of the sleeping period (5:30-7:00), which then decreased before breakfast. This peak constituted 55% of total BAs and consisted mostly of the primary BAs; CDCA and CA.

FGF19 circulating levels increase following the elevation of circulating glycine conjugated CDCA and DCA.

In this study we examined the relation between individual BAs and FGF19 using a cross-correlation analysis which is a measure of covariation of two signals in a time series. Our analysis indicated that FGF19 is well correlated with the glycine conjugates of CDCA and DCA with time lag of ~2 hours. Our data also indicate that the unconjugated BAs do not influence the level of FGF19 as supported by basal FGF19 night level despite a rapid increase of unconjugated BAs in serum.

Cholestyramine intake eliminates the diurnal rhythms of serum BAs, FGF19 and C4.

Interrupting the EHC by treatment with CME, with and without statin, stimulated BA synthesis as indicated by elevated C4 serum levels. While serum levels of CDCA, DCA and FGF19 were considerably reduced, serum CA stayed essentially unaltered. Moreover, the observed nightly peak of unconjugated BAs was not seen when CME was administered. The significant induction of BA synthesis by cholestyramine was slightly

attenuated by pretreatment with statin, though it stayed considerably higher compared to basal level.

Cholestyramine intake modulates the night levels of TG, glucose and insulin.

During the night we observed a moderate drop in the levels of TG, glucose and insulin in the basal state. Interestingly, this decline disappeared upon the administration of CME, and the levels of these metabolites tended to be higher than during basal state.

4.2 Paper II

An FXR agonist reduces bile acid synthesis independently of increases in FGF19 in healthy volunteers

Aim

This study aimed to evaluate the relative contribution of intestinal versus hepatic FXR signaling to suppress BA synthesis.

Findings

Bile acid synthesis occurs in the morning regardless of food intake.

Monitoring BA synthesis by measuring serum C4 level in this group indicated that the onset of BA synthesis occurs in the morning despite that the subjects were fasted. C4 serum levels continued to increase and peaked around noon after which it declined due to hepatic BA flux following food intake.

Suppression of BA synthesis can occur independently of FGF19 signaling during Px-102 intake.

When the single dose of Px-102 was administered, we observed a strong reduction (~80%) in C4 levels in all treatment groups including the subjects taken the lowest dose. However, FGF19 response to the drug was dose-dependent with no or minor changes at the lowest doses. Moreover, except at the highest doses, the decline in C4 levels preceded the increase in serum FGF19. Additionally, even after 24 hours, when FGF19 levels were normalized, C4 levels remained significantly suppressed. Based on this observation, we concluded that the suppression of BA synthesis in humans is primarily driven by BA-hepatic FXR signaling while intestinal FXR-FGF19 signaling might play a secondary role in this pathway.

Px-102 treatment induced fasting levels of serum BA.

Analysis of total and individual BAs indicated that, although not significant, there was an immediate dose dependent increase in their levels during the fasting state of the study (8 AM to 12AM). However, no clear change was seen during the postprandial state.

Px-102 intake reduced cholesterol synthesis and serum triglycerides

Since BA synthesis is directly linked to cholesterol synthesis, we monitored the changes in serum lathosterol, a marker of cholesterol synthesis. As expected, the levels of circulating lathosterol were significantly reduced when compared to controls, mainly at doses of 1.2 mg/Kg and higher. While there was no change in serum cholesterol, the level of TG tended to decline in response to high doses of Px-102; this reduction was mainly seen at night, however.

4.3 Paper III

Influence of food and sleep deprivation on bile acid metabolism in humans

Aim

In this study, we aimed to examine the effect of sleep and food deprivations on BA metabolism.

Findings

BA synthesis is reduced by prolonged fasting and remains unchanged by sleep deprivation.

C4 levels in food deprived subjects were considerably lower, and this lowering occurred already 18 hours after fasting; it was reduced by at least 50% after 66 hours of fasting. On the other hand, no clear changes in C4 level was detected during 55 hours of sleep deprivation. We also found that on the 8th day of the study, the C4 level was normalized.

Sleep deprivation suppresses FGF19 independently of BAs.

The only observed reduction in FGF19 serum levels was at 18 hours of fasting, after which its levels returned to normal values. FGF19 levels decreased significantly upon sleep deprivation. Interestingly, in both situations, changes in serum BAs were inconsistent with those of FGF19. As expected, BAs levels were lowered by prolonged fasting and unaffected by sleep deprivation.

Sleep deprivation has no major influence on lipid and BA metabolism.

TG levels and cholesterol synthesis monitored by serum lathosterol remained essentially unaltered during the 55 hours of sleep deprivation, possibly because of maintained regular food intake. In contrast, they were dramatically reduced by 66 hours of food deprivation.

4.4 Paper IV

Lowering of circulating FGF21 by modulation of bile acid metabolism in healthy males

Aim

To examine whether modulation of BA synthesis by treatment with cholestyramine/statin (paper I) or Px-102 (paper II) will influence the levels of serum FGF21.

Findings

FGF21 has a diurnal variation with increased nocturnal activity and basal daytime levels.

The levels of FGF21 in untreated (paper I) or placebo treated (paper II) subjects demonstrated that FGF21 has a diurnal rhythm characterized by a basal level during the day and a peak at late night to early morning. With more frequent night samplings, the nocturnal peak of circulating FGF21 was better displayed in the untreated subjects of study I. This was further supported by data from the placebo treated group, which - although nightly samples were not collected - demonstrated FGF21 morning levels (baseline and 24 hours samples) that were 2-fold higher than the day time levels.

Administration of cholestyramine or Px-102 reduces FGF21 serum levels.

Cholestyramine and Px-102 exerted opposing effects on BA synthesis. Nevertheless, both treatments were associated with a clear reduction in circulating levels of FGF21. Under these treatments, the nocturnal peak of FGF21 was eliminated pointing to a role of BAs in regulating FGF21 expression. The suppression in the levels of FGF21 was rapid and immediate when Px-102 was administered and this suppression persisted until 24 hours after drug intake.

5. DISCUSSION AND FUTURE PERSPECTIVES

The revitalized interest for the role of BAs in regulating lipid, BA and glucose metabolism has resulted in overwhelming amounts of research, with the aim both to understand their metabolism and to investigate their possible role as novel targets for the treatment of metabolic diseases. However, the majority of these studies have been conducted in mouse models, known to have unique BA composition and characteristics which complicates their translation into the human situation. This thesis contains several studies performed in healthy humans and presents a number of novel findings that should provide a foundation for future studies aimed at understanding human BA physiology and pathophysiology. The most important findings can be summarized as follows; a) In contrast to conjugated BAs, the diurnal variation of unconjugated BAs is characterized by a nocturnal peak, b) Changes in the diurnal levels of FGF19 are linked to conjugated but not unconjugated BAs, c) Feedback inhibition of BA synthesis can occur independently of FXR-FGF19 signaling, d) In conditions of sleep and food deprivation, circulating levels of FGF19 are controlled by BA-independent signaling and e) FGF21 circulating levels are directly influenced when BA synthesis is disturbed.

5.1 Normal diurnal variations

In Paper I and II, we were able to verify and strengthen results from previously conducted human studies on the normal diurnal variations of serum levels of total BAs, FGF19, serum markers of BA and cholesterol syntheses, and FGF21 [59, 83-87]. These analyses were based on samples collected from healthy male participants in study I (n= 8) and placebo-treated subjects in study II (n= 14).

BA and cholesterol metabolism

The observed postprandial appearance of BAs and FGF19 followed by declined C4 levels (Figure 4A, B, C and Figure 5) is in agreement with the concept that the transintestinal flux of BAs induces the secretion of FGF19 following activation of FXR [83]. Despite the highly efficient hepatic extraction of portal BAs [84, 88], postprandial rises still occur underscoring the pleiotropic effect of BAs in extrahepatic tissues [7]. Following the maximal hepatic inflow of BAs and FGF19, subsequent suppression in BA synthesis occurs. The increased C4 level in the morning confirms that BA production in humans has an intrinsic, meal-independent diurnal variation [59]. Asynchronous to the rhythm of BA synthesis [59], and consistent with earlier reports [59, 85, 89, 90], serum lathosterol in the untreated subjects showed a circadian rhythm characterized by a gradual increase during the evening and a peak between midnight and 4:00 AM (Figure 4D and Figure 5). These patterns are in marked contrast to those observed in mice and rats, where the diurnal changes in cholesterol synthesis occur in parallel with those of BAs.

Conjugated and unconjugated BAs

The diurnal rhythm of serum BAs has so far been mainly ascribed to total [59, 83] and conjugated BAs [59, 83, 91-93]. In paper I, we have uncovered a fundamental difference in the diurnal rhythms of the EHC of conjugated and unconjugated BAs. Thus, only the conjugated BAs peaked following meal intake while unconjugated BAs remained unaltered. Interestingly, the later rapidly and transiently increased at late night to early morning (5:30-7:00) during which the former stayed at basal level. Apparently, mice also exhibit a similar pattern of unconjugated BA peak during the day when their feeding activity is low [94]. Of interest is also that the levels of unconjugated BAs had returned to normal before breakfast. Interestingly, the presence of a similar nocturnal peak was also reported, however as a trivial observation, in 3 healthy volunteers by Steiner et al [91]. We herein report for the first time that unconjugated BAs have a distinct diurnal rhythm with a marked nocturnal peak.

Notably, this nocturnal peak is mostly caused by increased hepatic flux of CA and CDCA, with minor contribution from DCA which is mostly formed in the large intestine. Although this finding cannot be fully explained from the current study, the most plausible mechanism would be increased activity of deconjugating bacteria in the small intestine during sleep. Recent studies in rodents revealed that gut microbiota exhibit diurnal oscillations not only in their activity but also in the type of species and their tissue localization. These diurnal oscillations are controlled by food intake and diet composition. Accordingly, the intestine is exposed to different bacterial species during the day that impact the host physiology and metabolism, and contribute to body circadian activity [95, 96]. Therefore, in the light of these findings, our data potentially point to the presence of a diurnal variation in intestinal microflora in humans.

While further work is needed to provide firm conclusions about the physiological role of unconjugated BAs, we propose that their nocturnal appearance might have an influence on the central clock activity. This concept gains support from several lines of evidence from animal experiments, such as the ability of the unconjugated BAs to cross the blood brain barrier [97, 98] and the finding that the bacterial deconjugating enzyme, bile salt hydrolase (BSH) significantly alters the expression level of circadian clock genes through unconjugated BAs-dependent mechanisms [99]. Besides the proposed central effect of unconjugated BAs in humans, their relatively increased levels might suggest their contribution to regulate glucose and lipid metabolism during this inactive period. Such an effect is known to be induced by unconjugated BAs binding to TGR5 expressed in extrahepatic tissues [100, 101]. Investigating the effects of antibiotic treatment, and whether the unconjugated BA peak has any influence on BA, lipid or glucose metabolism will be of high interest.

Interestingly, the night rise in unconjugated BAs was not accompanied by any changes in FGF19 levels, in line with our finding that the circulating levels of FGF19 are only controlled by the transintestinal flux of conjugated BAs. The latter finding was confirmed by cross correlation analysis and Bayesian statistics that showed a strong time-lag correlation of ~2 hours between serum FGF19 and glycine conjugates of CDCA and DCA, and to a lesser extent CA. In contrast, no correlation was found between FGF19 and unconjugated BAs.

With these exciting, but still inconclusive findings regarding the unconjugated BAs, several questions remain unanswered which should provoke further investigations, such as: What is the role of the unconjugated BAs taken up by the liver at late night? Do they reach the brain and influence the hypothalamic-pituitary-adrenal axis? Is there any gender difference? Is this nocturnal peak affected by metabolic diseases such as obesity, dyslipidemia and diabetes? Is the nightly increase in unconjugated BAs linked to the reduced TG, glucose and insulin normally observed at this time period? Could in that case manipulation of unconjugated BA levels be a target for drug intervention in the treatment of metabolic disorders?

FGF21

Our investigations on patterns of rhythms in normal humans was also extended to that of FGF21, which were presented in paper III along with an exploration of a possible association between FGF21 and BA metabolism. To accomplish our aims we used samples obtained from experiments outlined in Papers I and II. Existing data about the presence of a circadian rhythm of serum FGF21 levels in humans were inconsistent [86, 87, 102, 103], and discrepancies were also reported among genders [103, 104]. Our analyses under basal conditions strongly support the previously expressed concept that there is a diurnal variation of circulating FGF21 in normal humans [86, 87], characterized by a lowering during the day and a remarkable increase at late night to early morning (~5:30) (Figure 4E and Figure 5). The expression of FGF21 has been found to be induced in prolonged energy-deprived conditions [70-72, 102], and has been associated with positive effects on insulin, glucose and TG homeostasis [76, 78, 105].

However, the presence of a 24-hour oscillation in circulating FGF21 suggests a metabolic regulatory effect elicited during non-starvation situations [106]. Whether FGF21 nocturnal activity is linked to the concomitant rise in unconjugated BAs or to decreased serum levels of TG, glucose and insulin during the current basal conditions (Figure 5) remains to be investigated. Like unconjugated BAs, FGF21 has also been found to cross the blood-brain barrier, and its metabolic effects are now presumed to be mediated through its action in the CNS [107, 108].

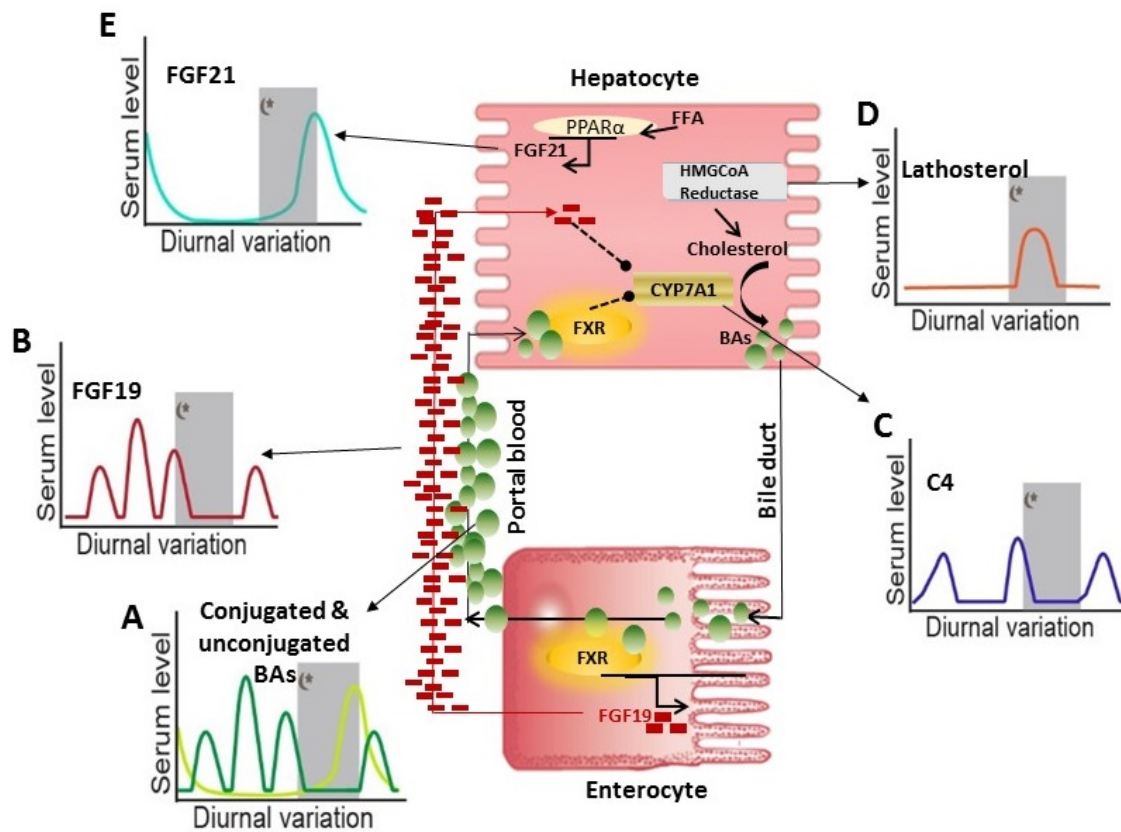


Figure 4. Summary of the normal diurnal variations in BAs, cholesterol and FGFs metabolism. Cholesterol synthesis monitored as lathosterol is more active after midnight. Asynchronous to that, BA synthesis marked by C4 increases twice a day, around noon and at night. Conjugated and unconjugated BAs have distinct circadian rhythms; conjugated BAs only increase postprandially and unconjugated BAs show one transient peak at late night to early morning. FGF19 circadian rhythm appears ~2 hours late of conjugated BA peaks. FGF21 has a diurnal variation similar to that of unconjugated BAs, a basal level during the day and strong increase at late night.

5.2 Perturbing BA synthesis by treatment with cholestyramine/statin and Px-102

The sections below discuss how the aforementioned normal diurnal variations are influenced by perturbing BA synthesis in healthy male subjects. In Paper I, the volunteers were treated with cholestyramine (CME) and cholestyramine combined with atorvastatin (CME+STAT) and in paper II, Px-102 was administered. In the later paper, the relative contribution of FGF19 vs BAs in the regulation of BA synthesis was investigated in response to Px-102 administration (Figures 5 and 6). Using these treatments we also explored the interrelationships among different players in BA and lipid metabolism.

BAs vs FGF19 in the regulation of BA synthesis following Px-102 administration

Most of the current evidence for an important function of FGF15/19 in inhibiting BA synthesis in the liver has been obtained from studies in transgenic and knockout mouse

models interfering with the FXR/FGF15/ β -Klotho/FGFR4 pathway [31, 36, 37, 41, 43], and from mouse studies where human FGF19 has been administered at very high doses [36, 109]. Circulating BAs, particularly the conjugated ones, are efficiently cleared by the human liver, thereby generating a considerable portal venous/systemic concentration gradient [84]. In contrast, no clear difference exists between portal and peripheral concentrations of FGF19 [110, 111]. This indicates that under physiological conditions, FGF19 levels reaching the liver are substantially lower than those needed to inhibit BA synthesis in experiments in vitro [47], or in humans infused with an FGF19 analog [46]. In papers I and II, we attempted to evaluate the relative contribution of BAs vs FGF19 in the regulation of BA synthesis in humans.

In paper I, we used different mathematical approaches through which the temporal changes in FGF19, different BA species and C4 during 32 hours were cross-correlated. Through this approach, we could demonstrate that conjugated BAs stimulate the secretion of FGF19. However, it was not possible to fully separate the effect of FGF19 from that of BAs regarding their influence on BA synthesis, due to their strong temporal relationship and to the fact that CYP7A1 activity is also governed by an intrinsic clock system that is independent of food intake [59, 112].

To further investigate the relative contribution of FGF19 and BAs to the regulation of BA synthesis, we performed analyses on samples from a pharmacokinetic study using the powerful FXR agonist, Px-102 (paper II). Our primary hypothesis was that the level of circulating FGF19 is the major regulator of CYP7A1 activity in humans, and we therefore predicted that treatment with Px-102 should first increase FGF19 which would then result in decreased serum C4 levels. We also predicted that both FGF19 and C4 should respond in a dose-dependent manner. The fact that no food was given until 4 hours after drug intake enabled exploring the response pattern of BA and cholesterol syntheses, individual BA levels and FGF19 without food interference.

As expected, FGF19 levels increased dose dependently in line with a similar response reported following the intraduodenal infusion of the natural FXR agonist CDCA in healthy humans [113]. While these findings, like ours, are fully consistent with the concept that circulating FGF19 is predominantly secreted from the small intestine, we cannot exclude that additional input, at least at the highest doses of FXR agonists, may have occurred from other organs such as the liver, as has been described in cholestatic conditions [114].

The results of this study did not support our original hypothesis, and would instead point to hepatic FXR activation is the key regulator of BA synthesis in this situation. This was based on several observations: Firstly, serum C4 levels showed that BA synthesis was maximally (80%) suppressed already at very low doses of Px-102, at which no significant change in the levels of FGF19 occurred. Secondly, the C4 response

to the FXR agonist was obvious already before the intake of the first meal, and was not preceded by increased levels of FGF19, except at high Px-102 doses. Thirdly, although FGF19 levels were normalized after 24 hours of Px-102 intake, C4 levels remained highly suppressed (Figure 5). A relatively minor contribution of the intestinal FXR pathway could be explained by the recent identification of a hepatic FXR target gene which codes for a protein capable of inducing rapid degradation of CYP7A1 mRNA in the liver [35]. Also in that work, the FGF15 inhibitory effect appeared as a late response. Whether human CYP7A1 mRNA transcripts are subject to a similar posttranscriptional regulation will be important to investigate.

Despite the above indications that signaling through the hepatic FXR is of major importance in the regulation of BA synthesis in humans, it cannot be excluded that FGF19 may exert important roles in this respect also in this species. Such an effect would also be in line with observations that mice infused with pharmacologic doses of, or made transgenic for, FGF19 have reduced BA synthesis. Also, infusing humans volunteers with a synthetic FGF19 variant resulted in a robust reduction of C4 levels [46]. However, considering the range of serum FGF19 levels under physiological conditions, the dose used to establish such a reduction was highly supraphysiological [115, 116]. Yet, a basal level of FGF19 may well be required to retain an intact inhibitory mechanism, even if the immediate feedback signaling is induced by FXR-agonistic BAs [47]. Such a mechanism would also be compatible with the mice experiments showing a marked increase in BA synthesis upon deletions of genes such as FXR, FGF15, FGFR4 and B-Klotho [31, 36, 37, 41, 43], as well as with the rapid and pronounced increases in C4 seen concomitantly with marked reductions of FGF19 in response to BA sequestration in humans [83, 115].

Effects of perturbing BA synthesis on BA metabolism

Acute treatment with cholestyramine, with or without statin, markedly attenuated (>50%) serum levels of total BAs. Because CME is known to preferentially bind the dihydroxy BAs, serum CDCA and DCA were highly blunted compared to CA [115, 117]. Of particular interest was the fact that the normally occurring nocturnal peak of unconjugated BAs disappeared during CME administration. In line with the concept that conjugated BAs stimulate FGF19 secretion, a parallel robust reduction (>90%) in FGF19 circulating levels was observed. The above effects were not enlarged upon pretreatment with statin (Figure 5).

Administration of CME clearly abolished not only the normal diurnal variation in the synthesis of BAs but also that of cholesterol. Following intake of the first dose of CME, the level of C4 and lathosterol increased up to 4-fold and 2-fold, respectively, compared to their baseline levels. However, atorvastatin pretreatment clearly attenuated this stimulation presumably reflecting a state of relative deficiency in hepatic precursor cholesterol. Nevertheless, serum levels of both C4 and lathosterol during CME+STAT

experiment remained significantly higher compared to the basal state. The effect of atorvastatin may not have reached its optimum, since only 2 days of pretreatment was used (Figure 5).

The changes in C4 and FGF19 levels during CME intake, along with data from animal experiments [36, 37, 41], would be compatible with the concept that the presence of a certain level of FGF19 may be necessary to maintain the normal mechanisms of feedback inhibition. In agreement with our previous cholestyramine experiment [115], it was clear that after cessation of CME intake, levels of C4 was normalized before circulating FGF19 levels had returned to baseline, and this was also observed when cholesterol synthesis was blocked by atorvastatin treatment. This raises the same question about the relevance of circulating FGF19 in the physiological regulation of BA synthesis in humans.

Interruption of the EHC by CME resulted in reduced serum BA levels. Somewhat unexpectedly, no major influence was seen on serum BAs following Px-102 administration. Studies in mouse models have revealed that FGF19 suppresses the activity of the apical sodium-dependent BA transporter (ASBT) that facilitates the ileal reabsorption of BAs [118, 119]. A similar effect, in addition to suppressed NTCP, occurred upon FXR activation [21, 32, 33, 48, 120]. Furthermore, FGF15/19 has been proposed to influence the enterohepatic circulation of BAs by inducing gallbladder relaxation and refilling [121, 122]. Based on all these findings, and that BA synthesis is suppressed following the administration of Px-102, we expected to see signs of a diminished pool of BAs. However, no such change was observed, instead, there was an apparent increase in serum BA levels during the fasting period of the study (8 AM to 12 AM) that, less distinctly, was also seen in the postprandial phase.

The observation of increased levels of serum BAs, particularly during the early (fasting) period indicates that the amount of circulating BAs was not reduced at this early stage of prominent synthesis inhibition. One possible explanation could be that the hepatic uptake of portal venous BAs by FXR-regulated transporters, such as NTCP, is rapidly reduced by FXR activation in the liver, a response reported in animal studies [120]. From inspection of the temporal patterns, there was no obvious relation to FGF19 levels. In contrast, when Px-102 was given to humans at a dose of 0.5 mg/Kg for 7 days, serum levels of BAs were reduced [123], and similar findings were reported following 7 days of treatment with the engineered FGF19 molecule mentioned earlier [46]. These findings also underscore that acute and chronic treatment with FXR agonists result in different responses in BA metabolism.

Effects of perturbing BA synthesis on lipid metabolism

Treatment with steroidal FXR agonists, such as CDCA and obeticholic acid suppresses BA synthesis and eventually reduces the hepatic demand for cholesterol and elevates

levels of total and LDL cholesterol [124-128]. These observations are the consequence of downregulation of hepatic LDL receptors and reduced LDL clearance, accompanied by a reduced HMGCoA reductase activity [61], Px-102 treated subjects had reduced lathosterol levels concurrent to those of C4. Interestingly, in contrast to what is observed following administration of the steroidal FXR agonist CDCA, total cholesterol was not changed following treatment with a single dose of Px-102. Similar findings were obtained when cynomolgus monkeys were fed 1 or 10 mg/Kg of Px-102 for up to 4 weeks [62], and longer-term treatment of mice with this compound was actually reported to result in decreased plasma cholesterol and TG [62, 63]. Non-steroidal FXR agonists may also stimulate transintestinal cholesterol excretion pathways in humans [129], a mechanism recently proposed based on studies in mice treated with Px-102 [63].

Treatment of humans with CDCA is also known to lower the secretion and concentration of TG in VLDL [130], presumably via hepatic FXR signaling [65, 131, 132]. Accordingly, we observed reduced postprandial TG levels in subjects given high doses of Px-102 in our study. Whether FGF19 is involved in this FXR-mediated regulation of lipid metabolism in humans is to be confirmed [115, 133], but administration of high doses of FGF19 lowers TGs in mice [134], an effect also seen in FGF19 transgene animals [135]. This effect seems to be a species-specific response to FGF19 in mice, however, and is not seen with FGF15 [45]. This is further supported by a similar reduction in TG levels in the CME experiment during which FGF19 was significantly eliminated. Since both Px-102 and CME administrations should cause diminished intestinal levels of BAs, the most plausible interpretation of the lower postprandial TG levels would be a reduced capacity of dietary fat absorption. The interesting effects of FXR agonists on lipoprotein metabolism require further studies, as well as their long-term responses in humans.

Some of the positive metabolic effects following FXR agonist treatment may be due to the increased circulating levels of FGF19, independent of the modulation of hepatic BA and cholesterol metabolism [47]. In agreement with that concept, recent studies in mice indicate that a major part of effects on body weight, insulin sensitivity and hepatic TGs observed in response to very high levels of circulating FGF19 are mediated by its actions in the central nervous system, similar to what is seen for FGF21 [136, 137].

A similar investigation in CME-treated subjects reported increased night levels of TG, glucose and insulin that were presumed to be mediated by a deficiency in BA pool caused by CME intake [115]. Although in our experiment no such elevations occurred, it was evident that the night decline in the levels of TGs, glucose and insulin observed under basal conditions was abolished by CME treatment. Whether these responses are linked to parallel changes in the EHC of unconjugated BAs at night cannot be confirmed in this study. A likely mechanism could be mediated by the enzyme fatty

acid transport protein 5 (FATP5) which is involved in the process of re-conjugation of the returned unconjugated BAs in the liver, and which also promotes uptake and activation of FFA [138, 139]. Hence, it is tempting to speculate that under normal situations a relative deficiency of FATP5 activity following a load of unconjugated BAs could result in reduced hepatic TG synthesis and improved glucose tolerance, in agreement with observations in FATP5 knockout mice [138, 139]. Conversely, when unconjugated BAs hepatic flux was abolished by CME intake, subsequent increases in TGs and glucose levels occurred. An unexpected observation here was that CME+STAT treatment did not further enhance the night increase of TGs levels despite of the reduced hepatic cholesterol stores and hence rate of BA synthesis. This may be related to the fact that statins also reduce endogenous VLDL production [140]. Further studies in suitable animal and human models, including genetic diseases of BA conjugation [141] and hypertriglyceridemia with BA overproduction [142] will be important to better understand the metabolic links between BA, glucose and TG metabolism.

Effects of perturbing BA synthesis on FGF21

FGF21 has been proposed as a potential metabolic regulator in conditions of obesity and diabetes both in humans and animals [76, 143, 144]. The correlation between serum FGF21 and BA synthesis (measured by C4) was previously investigated in a cohort of 75 healthy subjects, in which no correlation was detected [102]. Interestingly, recent animal investigations indicated a direct influence of FGF21 on BA synthesis [79-81], although the presented effects in these studies are somewhat contradictory. The possibility that variations in the EHC of BAs and FXR activity might influence FGF21 levels [145] lead us to explore this further. We considered our well-designed CME and Px-102 experiments to represent a reliable and useful approach to be used for this purpose. Knowing that CME and Px-102 exert opposing effects on BA synthesis, we expected FGF21 to respond differently to these drugs. Nevertheless, we observed a clear decline in circulating levels of FGF21 in both situations, and a further reduction in response to CME+STAT treatment. Both treatments abolished the nocturnal/morning rise of FGF21 (Figure 5), suggesting an existing association between BAs and FGF21 in humans. An interesting observation was that immediately after Px-102 intake, there was a rapid reduction in FGF21 levels already at the lowest dose, and FGF21 remained lowered until 24 hours after drug intake (Figure 5). Thus, our observations are not in line with data from animal models that showed increased FGF21 expression in conditions of reduced BA EHC and increased FXR activity [145-147]. Moreover, our Px-102 data with significantly elevated FGF19 and the fact that reduced FGF21 levels always preceded FGF19 increase, do not agree with the finding that FGF19 stimulates FGF21 gene expression [145].

It has been proposed that the favorable metabolic effects of FGF21 could be partly mediated by BA-dependent signaling [79, 145]. Hence, a likely mechanism for FGF21 lowering by CME and Px-102 treatments would be related to a reduced BA signaling in the liver. While CME treatment clearly disrupted the BA EHC, there was a rapid dose-dependent increase in serum BAs upon Px-102 intake. As mentioned, the latter effect could be the consequence of reduced hepatic uptake of BAs due to FXR-mediated suppression of NTCP [120]. This, along with the rapid inhibition of CYP7A1 by Px-102, would eventually reduce hepatic levels of BAs, a situation resembling what occurs following continued CME treatment. In agreement with this reasoning, pretreatment with statin - which blocked the capacity to increase BA production from cholesterol - resulted in a more pronounced lowering of FGF21. An opposing situation may explain why serum FGF21 levels were markedly higher, and correlated with serum BA levels, in patients with cholestatic liver disease [147].

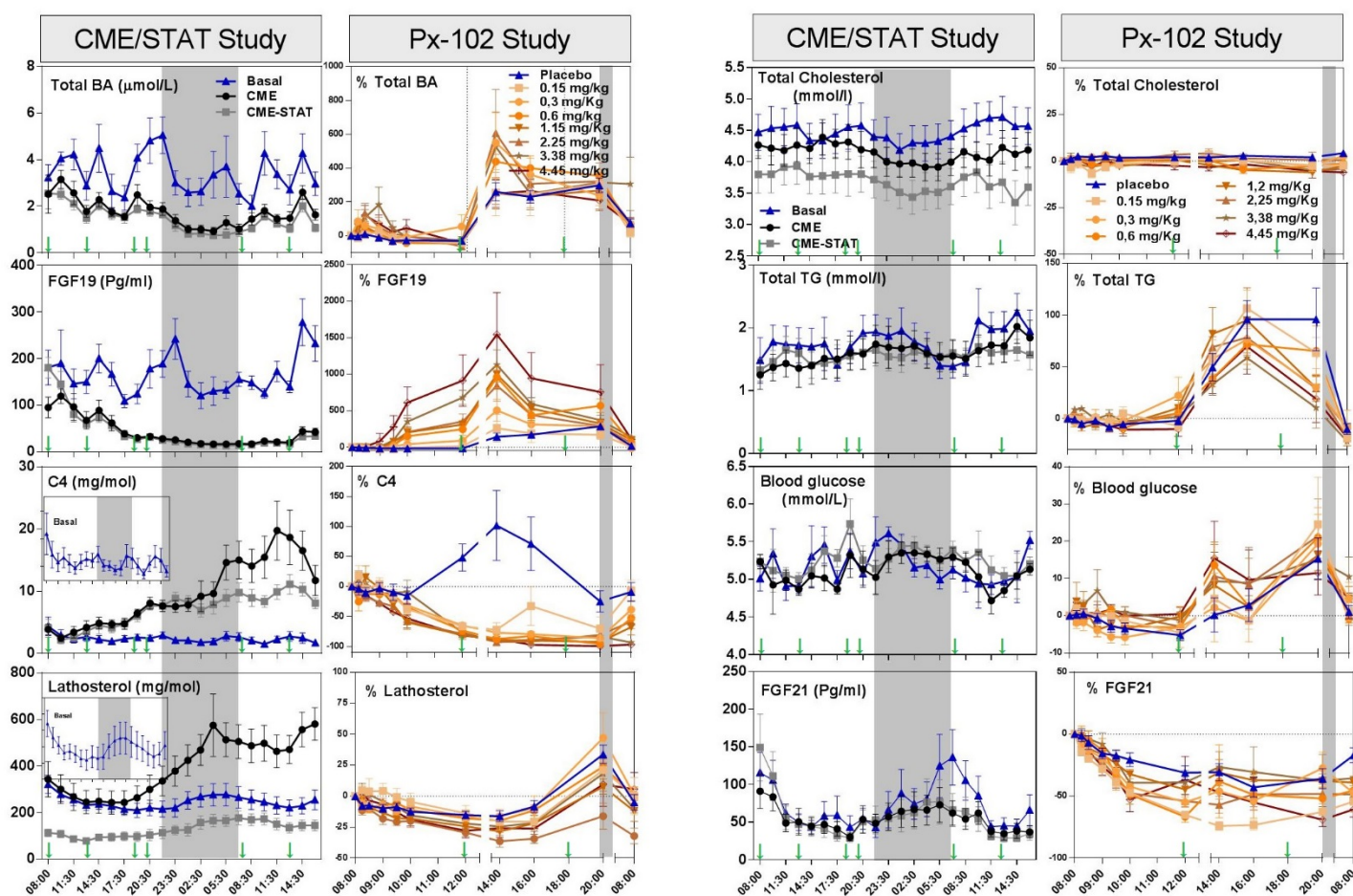


Figure 5. Diurnal variations of serum levels of BAs, FGF19, markers of cholesterol and BA syntheses, total cholesterol, TG, glucose and FGF21 under normal conditions (blue) and during treatment with CME (black), CME-STAT (gray) and Px-102 (colored). Data obtained from Px-102 study are presented as % change from baseline. Green arrows indicate meals intake.

It is noteworthy that some of the effects elicited by FXR synthetic agonists are not seen, or may be opposite to those induced by natural agonists. To establish whether a similar effect on FGF21 is obtained by treatment with natural FXR agonists - such as CDCA [61] - or by chronic treatment with synthetic FXR agonists will require additional investigations.

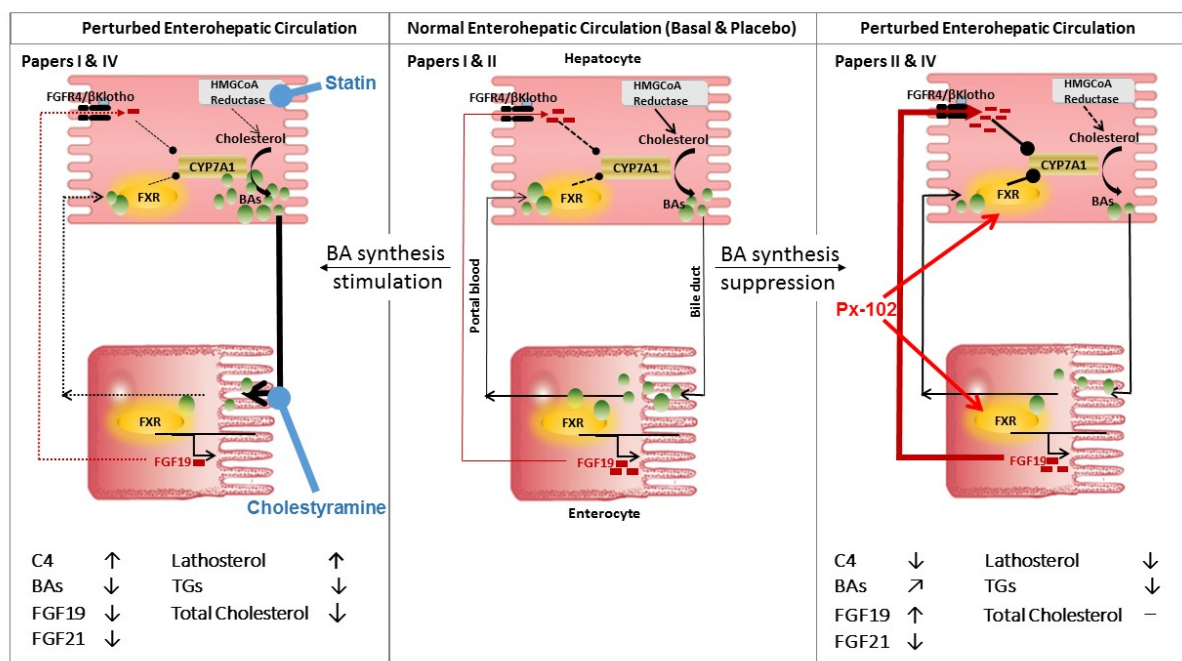


Figure 6. Summary of the effects of perturbing BA synthesis. Stimulation of BA synthesis by cholestyramine with/without statin increases cholesterol synthesis but decreases BAs, FGF19, FGF21, total cholesterol and TGs serum levels (Paper I & IV). Treatment with Px-102 suppresses BA and cholesterol syntheses, decreases TGs and FGF21, increases FGF19 and somehow BAs serum levels.

5.3 Effect of sleep and food deprivation on BAs and lipid metabolism

The circadian rhythms of BA and lipid metabolism are a consequence of synchronization between peripheral and central nervous signals that are controlled by feeding/fasting and light/dark cycles. Desynchronization of these circuits increases the prevalence of metabolic disorders such as obesity and diabetes. In paper IV we investigated the effect of disturbing these cycles on major players of BA and lipid metabolism in healthy humans. This study highlights a number of novel observations that may call for revising some established concepts in the regulation of synthesis and the EHC of BAs. The main results were: (A) During food deprivation, BA synthesis is not related to FGF19 and BA serum levels, (B) Changes in the circulating levels of FGF19 during prolonged fasting and sleep deprivation are independent of serum BA levels, (C) Sleep deprivation is associated with markedly suppressed FGF19 levels.

Changes in BA and cholesterol synthesis during food and sleep deprivation

We made our hypothesis based on the well-defined concept that FGF19 is produced postprandially in response to the transintestinal flux of BAs [83, 148], whereas during food deprivation when the EHC of BAs is inactive, no FGF19 is secreted. Subsequently, the absence of these two inhibitory signals should stimulate BA synthesis under this situation. However, the progressive strong decline in C4 levels that was observed during fasting was not related to serum BAs, the levels of which were reduced. Serum FGF19 remained essentially unaltered, except for at 18 hours. During both prolonged fasting and sleep deprivation, changes in C4 and lathosterol were closely related. It is known that during fasting, the liver capacity to synthesize cholesterol is suppressed, partly due to lack of activation of sterol regulatory element-binding protein-2 (SREBP2) [149-152]. Such an effect would eventually result in a reduced rate of BA synthesis, a concept also gaining some support from the fact that both lathosterol and C4 levels returned to normal when food intake was commenced during sleep deprivation.

Changes of circulating FGF19 levels during food and sleep deprivation are BA independent

A remarkable finding in this study was the non-synchronized changes in BA and FGF19 levels during both food and sleep deprivation. The progressive drop in serum FGF19, during sleep deprivation, despite of normal food intake and maintained EHC of BAs is surprising. This finding suggests the presence of additional mechanisms involve in regulating FGF19 secretion under this very stressful condition, when the brain master clock function is clearly disturbed. This raises the interesting possibility of a central regulation of intestinal FGF19. Taking into consideration that much evidence now supports the concept that FGF19 and FGF21 may exert much of their metabolic effects on the hypothalamic level [76, 153, 154], it should be of great interest to explore this further.

6. CONCLUSIONS

Our experiments provide an ample characterization of the normal diurnal variation of the EHC of BAs, and of how its manipulation may influence human metabolism. They also underscore the importance of the sleeping period in eliciting metabolic changes, and provide a foundation regarding the impact of feeding, fasting and biological clocks on BA and FGF19 metabolism in man. Moreover, the presented studies further expand the current understanding of the importance of BAs in physiology and metabolic signaling. This is not only of significance from a basic science standpoint, but should also provide a foundation for later development of diagnostic and therapeutic principles regarding highly prevalent diseases such as dyslipidemia, diabetes, fatty liver and cardiovascular disease.

The new findings of the presented work can be summarized as following;

- 1) In humans there are two independent EHCs of conjugated and unconjugated BAs. Only conjugated BAs seem to induce FGF19 secretion and suppress BA synthesis. There is a difference between individual BAs in this regulation. Finally, our data confirm and extend established concepts of the regulation of BA and lipid metabolism.
- 2) Under treatment with a non-steroidal FXR agonist, such as Px-102, feedback inhibition of BA synthesis is elicited by hepatic FXR signaling without the involvement of FGF19 as an early response.
- 3) In conditions of sleep and food deprivation, changes in circulating levels of FGF19 were not related to those in BA levels, suggesting the involvement of additional (central) mechanisms in the regulation of FGF19 levels
- 4) Circulating FGF21 levels are reduced when BA synthesis is modulated, presumably as a consequence of reduced intrahepatic BA levels.

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